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Comparison of Four Markers for Quantifying Microbial Protein Flow from the Rumen of Lactating Dairy Cows*

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ABSTRACT

Eight ruminally cannulated lactating cows from a study on the effects of dietary rumen degraded protein (RDP) on production and N metabolism were used to compare ^{15}N, total purines, amino acid (AA) profiles, and urinary excretion of purine derivatives (PD) as microbial markers for quantifying the flow of microbial protein at the omasal canal. Dietary RDP was gradually decreased by replacing solvent soybean meal and urea with lignosulfonate-treated soybean meal. The purine metabolites xanthine and hypoxanthine were present in digesta and microbial samples and were assumed to be of microbial origin. The sum of the purines and their metabolites (adenine, guanine, xanthine, and hypoxanthine) were defined as total purines (TP) and used as a microbial marker. Decreasing dietary RDP from 13.2 to 10.6% of dry matter (DM) reduced microbial nonammonia N (NAN) flows estimated using TP (from 415 to 369 g/d), ^{15}N (from 470 to 384 g/d), AA profiles (from 392 to 311 g/d), and PD (from 436 to 271 g/d). Averaged across diets, microbial NAN flows were highest when estimated using TP and ^{15}N (398 and 429 g/d), lowest when using PD (305 g/d), and intermediate when using AA profiles (360 g/d) as microbial markers. Correlation coefficients between ^{15}N and TP for fluid-associated bacteria, particle-associated bacteria, and total microbial NAN flows were 0.38, 0.85, and 0.69, respectively. When TP was used as the microbial marker, ruminal escape of dietary NAN was not affected by replacing solvent soybean meal with lignosulfonate-treated soybean meal in the diets. The direction and extent of response of dietary and microbial NAN flow to dietary treatments were similar when estimated using ^{15}N, AA profiles, and PD, and were in agreement with previously published data and National Research Council predictions. Microbial and dietary NAN flows from the rumen estimated using ^{15}N appeared to be more accurate and precise than the other markers. Caution is required when interpreting results obtained using TP as the microbial marker.
(**Key words:** microbial marker, ^{15}N, purines, dairy cow)

Abbreviation key: A = adenine, **APE** = atom percent excess, **FAB** = fluid-associated bacteria, **FP** = omasal fluid phase, **G** = guanine, **HX** = hypoxanthine, **LP** = omasal large particle phase, **NANMN** = nonammonia non-microbial N, **^{15}NB** = ^{15}N background, **OTD** = omasal true digesta, **PAB** = particle-associated bacteria, **PD** = purine derivatives, **SP** = omasal small particle phase, **TP** = total purines, **X** = xanthine.

INTRODUCTION

The high-quality microbial protein synthesized in the rumen of dairy cows contributes the majority of total AA flowing to the small intestine (Clark et al., 1992). However, inefficient use of degraded protein by ruminal microbes results in conversion of substantial amounts of dietary AA-N into ammonia-N that, if not incorporated into microbial protein, is eventually excreted as urea and may be lost to the environment (Broderick et al., 1991). Optimization of microbial protein synthesis in the rumen should increase feed N efficiency while reducing losses. However, studies on the factors affecting microbial yield and efficiency rely on accurate measurement of microbial protein synthesis. Among several markers, the total purines (**TP**) procedure of Zinn and Owens (1986), with modifications (Ushida et al., 1985; Aharoni and Tagari, 1991), and ^{15}N-ammonium salts, have been used most extensively to estimate microbial protein yield. Although low recoveries of TP have been reported when using the spectrophotometric method of Zinn and Owens (1986; Makkar and Becker, 1999; Obispo and Dehority, 1999), recent modifications of this method (Makkar and Becker, 1999; Reynal et al., 2003) substantially improved purine recovery. Moreover, the direct quantification and complete recovery of adenine (**A**) and guanine (**G**) using an HPLC method resulted in microbial protein flows that were highly correlated

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(R = 0.94) to those estimated with the modified Zinn and Owens method (Reynal et al., 2003). Theoretically, the external marker ^{15}N offers several advantages over TP. The ^{15}N should be evenly distributed throughout the microbial cell; thus, microbial lysis during isolation, although not affecting ^{15}N enrichment, would result in loss of purines from cell cytoplasm, lowering the purine:N ratio and leading to overestimation of microbial flows (Martin-Orué et al., 1998; Carro and Miller, 2002). Moreover, ^{15}N-labeled protein leaving the rumen that was enriched in excess of natural abundance will only be of microbial origin, whereas a portion of the purines leaving the rumen may be of dietary origin (Pérez et al., 1996b; Vicente et al., 2004). However, results from comparisons between TP and ^{15}N reported in the literature have been inconsistent. The use of TP resulted in higher (Carro and Miller, 2002), lower (Firkins et al., 1987b; Pérez et al., 1996a), or similar (Calsamiglia et al., 1996; Pérez et al., 1997a) estimates of microbial yield compared with ^{15}N.

Both markers give marker:protein ratios that differ between bacteria associated with fluid and particle phases (Craig et al., 1987; Firkins et al., 1987a), and between bacteria and protozoa (Hristov and Broderick, 1996). Thus, the marker:N ratios and the relative contribution to total microbial flow of fluid- and particle-associated bacteria and protozoa should be accounted for when estimating microbial flows. Although the omasal sampling technique allows for the isolation and measurement of flow of bacteria associated with liquid and particle phases, lack of specific markers precludes accurate assessment of the protozoal contribution to microbial protein flow. Shabi et al. (2000) used linear programming based on the AA profiles of isolated bacteria and protozoa, plus that of dietary protein and endogenous protein, to estimate the relative contributions from these 4 sources to total AA flow from the rumen. An approach of this type may not be influenced by factors altering marker:protein ratio and could be more reliable for estimating AA contributions from all sources. This method relies on accurate and precise AA analyses and digesta flow estimates, on representative microbial isolations, and on the assumption that the AA profile of RUP is the same as that of dietary protein. However, the AA profiles of RUP of feedstuffs incubated in situ were significantly altered by ruminal degradation (Erasmus et al., 1994; O'Mara et al., 1997).

Alternatively, microbial protein flow from the rumen can be estimated from the urinary excretion of purine derivatives (**PD**) using published equations relating PD excretion to microbial flows from the rumen (Chen et al., 1990; Balcells et al., 1991; Vagnoni et al., 1997; Orellana Boero et al., 2001). This noninvasive approach is based on the principle that most of the urinary PD are derived from microbial nucleic acids flowing out of the rumen. However, the extent of the endogenous contribution to urinary PD and the proportions of total PD excreted through nonrenal routes may vary depending on the nutritional and physiological status of the animal (Chen et al., 1990; Balcells et al., 1991). Therefore, the use of equations developed using animals under experimental conditions that differ substantially from those of the study where they are applied (e.g., dry vs. lactating cows) may result in biased estimates of microbial flow.

The objective of this study was to compare the use of TP, ^{15}N, AA profiles, and PD as microbial markers for quantifying microbial protein flows at the omasal canal of dairy cows fed diets differing in protein concentration and ruminal degradability.

MATERIALS AND METHODS

Experimental Procedure

Eight ruminally cannulated cows that were part of a larger trial studying the effect of dietary RDP on production and N metabolism (Reynal and Broderick, 2005) were assigned to a replicated 4 × 4 Latin square with 28-d periods. Cows were fed TMR containing (DM basis) 37.1% corn silage, 12.7% alfalfa silage, 0.9% vitamins and minerals, and 49.3% concentrate mix. The concentrate was adjusted to provide the same amount of CP from solvent soybean meal and lignosulfonate-treated soybean meal (SoyPass, LignoTech USA, Inc., Rothschild, WI), and decreasing amounts of urea, to obtain 4 different RDP concentrations (as % of diet DM): diet A (13.2% RDP), diet B (12.3% RDP), diet C (11.7% RDP), and diet D (10.6% RDP). Diet compositions are summarized in Table 1. Care and handling of the experimental animals including ruminal cannulation was conducted as outlined in the guidelines of the University of Wisconsin institutional animal care and use committee. Other details of the feeding trial are described in the companion paper (Reynal and Broderick, 2005).

The omasal sampling technique developed by Huhtanen et al. (1997), as modified by Ahvenjärvi et al. (2000), was used to quantify digesta flow from the rumen. Indigestible NDF (Huhtanen et al., 1994), $YbCl_3$ (modified from Siddons et al., 1985), and CoEDTA (Udén et al., 1980), which were mainly associated with the large, small, and fluid phases of digesta, respectively, were used as flow markers at the omasal canal. Before starting the infusion of markers, a sample of whole ruminal contents was taken from each cow in each period and stored at –20°C for later determination of ^{15}N background (^{15}**NB**). Cobalt-EDTA, $YbCl_3$, and 10% atom excess $^{15}NH_4SO_4$ were dissolved in distilled water and infused into the rumen at constant rates of 2.6 g of Co,

Table 1. Composition of diets.

Item	Diet A	B	C	D
Ingredients[1]	(% of DM)			
Corn silage	37.1	37.1	37.1	37.1
Alfalfa silage	12.7	12.7	12.7	12.7
Rolled HMSC	32.4	32.1	31.9	31.7
Solvent SBM	16.43	10.95	5.48	—
LSBM	—	5.87	11.74	17.61
Urea	0.50	0.33	0.17	—
Vitamins and minerals	0.90	0.90	0.90	0.90
Nutrient content of diets				
CP, % of DM	18.8	18.3	17.7	17.2
Non-urea N, % of DM	2.77	2.77	2.75	2.75
RDP,[2] % of DM	13.2	12.3	11.7	10.6
RUP,[3] % of DM	5.8	6.2	6.0	6.6
NFC,[4] % of DM	49.2	49.9	50.6	51.4

[1]HMSC = High-moisture shelled corn, LSBM = lignosulfonate-treated SBM.

[2]Least squares means of RDP measured in vivo as: RDP, % of DM = (Total CP intake, kg/d − omasal RUP flow, kg/d) × 100/DMI, kg/d.

[3]Least squares means of RUP measured in vivo as: RUP, % of DM = (Total omasal CP flow, kg/d − microbial CP flow, kg/d) × 100/DMI, kg/d.

[4]Predicted by the NRC (2001) model.

2.2 g of Yb, and 182 mg of ^{15}N per d in 3.12 L of solution. Markers were continuously infused for 158 h starting at 0900 h on d 20 to 2300 h on d 26 using 2 syringe pumps (model 33; Harvard Apparatus, Inc., Holliston, MA). Using the omasal sampling technique, spot samples of digesta were collected from the omasal canal at 1000, 1100, 1200, 1300, 1400, 1500 on d 23 and d 25, and 1600, 1700, 1800, 1900, 2000, and 2100 h on d 24 and d 26, such that the samples taken represented two 12-h feeding cycles, each over 2 d (d 23 to 24 and d 25 to 26 for the first and second feeding cycles, respectively). On each sampling day from each cow, 285-mL omasal spot samples were taken at each of the first 5 sampling times and a 785-mL spot sample taken at the last sampling time. Subsamples of 85 mL at all 6 sampling times were pooled and held in an ice-bath to yield one 510-mL composite from each cow on each sampling day. Subsamples of 200 mL from all 12 sampling times were pooled and held at –20°C as they were collected to yield two 2.4-L omasal composites, corresponding to sampling d 23 to 24 and 25 to 26 for each cow in each period. The 2.4-L omasal composites were stored at –20°C until processed.

The balance of the last subsample (500 mL) from each cow on each day was processed immediately after collection to isolate protozoa based on the procedures described by Hristov et al. (2001). Subsamples were squeezed through 2 layers of cheesecloth, and retained solids were washed with 500 mL of McDougall's buffer (at 39°C) containing 2.5 g of glucose and 0.25 g of cysteine-HCl. Filtrates from the original subsample and the buffer wash were transferred to a 1-L separatory funnel that was placed in a waterbath at 39°C for 45 min. Bacterial fermentation resulted in the formation of a buoyant upper phase of feed particles, allowing protozoa to sediment and form a distinct layer at the bottom of the separatory funnel. The protozoal sediment was carefully drawn off, layered on top of 20 mL of 30% (wt/vol) sucrose solution and centrifuged (150 × *g* for 5 min at 4°C). The resulting pellet was washed 3 times with 0.85% (wt/vol) NaCl solution followed by centrifugation (1200 × g for 5 min at 4°C), and stored at –20°C until later analysis.

The 2.4-L pooled omasal composites were thawed at room temperature and separated into 3 digesta phases as follows. Samples were squeezed through 1 layer of cheesecloth, and the retained solids were defined as the omasal large-particle phase (**LP**). The filtrate was centrifuged at 1000 × *g* (5°C, 5 min), and the supernatant was carefully decanted from the pellet. The supernatant was defined as the omasal fluid phase (**FP**) and the pellet was defined as the omasal small particle phase (**SP**). The separated phases were frozen, freeze-dried, and ground through a 1-mm screen (Wiley mill; Arthur H. Thomas, Philadelphia, PA).

At the end of each sampling day, the 510-mL omasal composite from each cow was squeezed through 1 layer of cheesecloth; solids retained on the cheesecloth were washed with 400 mL of 0.85% (wt/vol) NaCl solution and squeezed again. Filtrates (equivalent to the FP) were pooled, held on ice, and processed approximately 4 h after collection for isolation of fluid-associated bacteria (**FAB**). Following a procedure described by Whitehouse et al. (1994), the solids retained on the cheesecloth (equivalent to LP phase) were transferred to a 500-mL bottle; 350 mL of cold (5°C) solution containing 0.85% (wt/vol) NaCl and 0.1% (vol/vol) Tween 80 solution was added. Bottle contents were mixed thoroughly and held on ice until processed for isolation of particle-associated bacteria (**PAB**). Filtrates for FAB isolation were centrifuged (1000 × *g*, 5°C, 5 min) and the pellets were held at 5°C. Supernatants were carefully decanted and centrifuged again (11,300 × *g*, 5°C, 30 min); these supernatants were decanted and discarded. Pellets were resuspended in 100 mL of McDougall's buffer and recentrifuged (11,300 × *g*, 5°C, 30 min) and the resulting FAB pellets were stored at –20°C. The pellet saved from the 1000-×-*g* centrifugation step (equivalent to the SP) was mixed with the contents of the 500-mL bottle saved for PAB isolation, blended for 20 s in a single-speed Waring blender (Waring Products Division, New Hartford, CT), transferred back to the bottles and held at 5°C for 24 h. The blended contents were then squeezed through 2 layers of cheesecloth and

the filtrate was centrifuged (1000 × g, 5°C, 5 min); supernatants were carefully decanted and centrifuged again (11,300 × g, 5°C, 30 min). Supernatants were decanted and discarded, and pellets were resuspended in 100 mL of McDougall's buffer and recentrifuged (11,300 × *g*, 5°C, 30 min). The resulting PAB pellets were stored at –20°C.

Indigestible NDF was determined as follows in LP, SP, and TMR samples (but not FP; Ahvenjärvi et al., 2000). Samples (1 g for LP and TMR; 4 g for SP) were weighed into duplicate 5- × 10-cm Dacron bags with a pore size of 6 μm, incubated in the rumens of 2 cows for 12 d, removed from the rumen and rinsed with water, then subjected to conventional NDF analysis (Hintz et al., 1995). For Co and Yb analysis, 1-g samples of SP and LP and 0.5-g samples of FP were ashed at 500°C for 16 h and then solubilized in 15 mL of concentrated HCl and a solution of 0.6% (wt/vol) LiOH to a final mass of 100 g. Samples of the markers infused (0.5 mL) were diluted with 15 mL of concentrated HCl and 84.5 mL of LiOH solution before analysis. Concentrations of Co and Yb were analyzed by direct current plasma emission spectroscopy (Combs and Satter, 1992; SpectraSpan V; Fison Instruments, Valencia, CA). Based on concentrations of Co, Yb, and indigestible NDF in LP and SP, and of Co and Yb in FP, DM from freeze-dried FP, SP and LP were recombined in the correct proportions to reconstitute the omasal true digesta (**OTD**) flowing out of the rumen based on the triple-marker method of France and Siddons (1986). Aliquots of SP and LP phases were mixed in the correct proportions based on the markers to yield a 2-g sample that was ground through a 0.5-mm screen with a Udy Cyclone Sample mill (Udy Corporation, Fort Collins, CO) and defined as small plus large particles (**SP+LP**).

The OTD samples were analyzed for total N using a combustion assay (Leco FP-2000 N Analyzer; Leco Instruments, Inc., St. Joseph, MI). Extracts were prepared from weekly composites of TMR and from OTD samples in distilled water (Muck, 1987) as follows: 10 mL of pH 2.2 Na-citrate buffer was added to 0.5 g of sample; after mixing, samples were held at 39°C for 30 min, and centrifuged (15,000 × *g*, 4°C, 15 min). The supernatant was stored at –20°C for later analysis. These extracts were centrifuged (15,000 × *g*, 4°C, 15 min) and analyzed for ammonia (Reynal and Broderick, 2003) using flow-injection analysis (Dual-Channel QuikChem 8000 FIA, Lachat Instruments, Milwaukee, WI).

Frozen samples for ruminal ^{15}NB determination and those of FAB and PAB were freeze-dried, then sequentially ground through a 1-mm screen (Wiley) and 0.5-mm screen with an Udy Cyclone Sample mill (Udy Corporation). Two composites each of FAB and PAB were prepared by mixing equal DM from d 23 and d 24, and from d 25 and d 26, for each cow in each period. Each bacterial composite sample represented one 12-h feeding cycle. Samples of OTD, FAB, PAB, protozoa, and TMR were hydrolyzed in 6 *N* HCl containing 0.5 m*M* NorLeu and 1 g/L of phenol without pretreatment (Gehrke et al., 1985; Nagel and Broderick, 1992) or after oxidation with performic acid (for Met and Cys; Elkin and Griffith, 1985). Hydrolysates were analyzed for individual AA using NorLeu as an internal standard by ion-exchange chromatography with ninhydrin detection (Beckman 6300 Amino Acid Analyzer, Beckman Instruments, Inc., Palo Alto, CA) as described in the companion paper (Reynal and Broderick, 2005). The OTD, FAB, PAB, protozoa, and TMR samples were also analyzed for Trp using an HPLC method adapted from Delhaye and Landry (1986) and Landry and Delhaye (1992) and using 5-methyltryptophan as an internal standard. Other details on Trp analysis and recovery are also described in the companion paper (Reynal and Broderick, 2005).

Direct determination of A and G in samples of FP, SP+LP, PAB, FAB, and protozoa was performed using the HPLC method of Balcells et al. (1992) as modified by Makkar and Becker (1999). Acid hydrolysis was done using 2 *M* $HClO_4$. Allopurinol and caffeine were used as internal standards. The HPLC system used was as described by Reynal et al. (2003). Recovery of purines was estimated by carrying Torula yeast RNA through the assay. Samples of Torula yeast alone, and of FP, SP+LP, FAB, PAB, and protozoa were analyzed for purines, with and without addition of known amounts of Torula yeast RNA, to determine purine recoveries. Peak purity was assessed by comparing the UV scans (220 to 350 nm) of eluant peaks corresponding to A and G in 4 samples each of FP, SP+LP, FAB, PAB, and protozoa samples to scans of eluant peaks from the injection of pure A and G.

Samples of ^{15}NB, FAB, PAB, protozoa, FP, and SP+LP (100 μg of N) were weighed into 5 × 9-mm tin capsules and pH was increased to 12 by addition of 50 μL of 72-m*M* K_2CO_3. Capsules were placed in a 60°C oven for 24 h to volatilize ammonia. Samples were analyzed for NAN and ^{15}N atom percent excess (**APE**) in NAN by isotope ratio mass spectrometry (Stable Isotope Facility, Department of Agronomy and Range Science, University of California-Davis).

On d 22 of each period, 2 spot urine samples were collected by vulval stimulation from all cows at 0400 and 1600 h, corresponding to 18 and 6 h after feeding, respectively. Urine samples were immediately acidified after collection by diluting 1 volume of urine with 4 volumes of 0.072 *N* H_2SO_4, and were stored at –20°C. Later, urine samples were thawed at room temperature

and filtered through Whatman no. 1 filter paper. Filtrates were analyzed for creatinine using a picric acid assay (Oser, 1965) adapted to a flow-injection analyzer, for allantoin using the method of Vogels and van der Grift (1970) adapted to a 96-well plate reader, and for uric acid using a commercial kit (No. 1830-200, Thermo DMA, Arlington, TX).

Marker Calculations

Nonammonia N in omasal samples was calculated by difference between total N and ammonia N. Based on the similar background ^{15}N in microbes and digesta observed by Ahvenjärvi et al. (2002), the ^{15}NB used to compute ^{15}N APE in both bacterial and digesta fractions was defined as the ^{15}N content of ruminal samples before infusion in each period. The mean ^{15}NB was 0.3677 (SD 0.0002) atom % ^{15}N over all 4 periods. The ^{15}N-APE (above natural background) was calculated for digesta and microbial samples from each cow in each period as follows:

$$^{15}\text{N-APE} = {}^{15}\text{N-atom \%} - {}^{15}\text{NB}$$

Assuming that isolated FAB and PAB were representative of the bacterial biomass flowing with the FP and the SP+LP phases, respectively, the FAB-NAN and PAB-NAN flows into the omasal canal were calculated using TP and ^{15}N as microbial markers. The following calculations were used when ^{15}N was the microbial marker:

$$\text{FAB-NAN flow} = \text{FP flow} \times \text{NAN in FP} \times ({}^{15}\text{N APE in FP}/{}^{15}\text{N APE in FAB})$$

$$\text{PAB-NAN flow} = (\text{SP flow} \times \text{NAN in SP} + \text{LP flow} \times \text{NAN in LP}) \times ({}^{15}\text{N APE in SP+LP}/{}^{15}\text{N APE in PAB}),$$

where flows are in g/d and NAN contents in g/g. The calculations used when TP was the microbial marker were as follows:

$$\text{FAB-NAN flow} = \text{FP flow} \times \mu\text{moles TP/g FP} \times \text{g NAN}/\mu\text{mol TP in FAB}$$

$$\text{PAB-NAN flow} = \text{SP+LP flow} \times \mu\text{moles TP/g SP+LP} \times \text{g NAN}/\mu\text{mol TP in PAB},$$

where flows are in g/d. Other calculations are described in the companion paper (Reynal and Broderick, 2005).

Daily urine volume and excretion of PD (allantoin plus uric acid) were estimated from urinary creatinine concentration and BW assuming a creatinine excretion rate of 29 mg/kg of BW (Valadares et al., 1999). Microbial NAN flow from the rumen was estimated based on the excretion of PD using the equation of Vagnoni et al. (1997) and the purine:N ratio in FAB plus PAB isolates in their proportions flowing at the omasal canal.

Proportions of N fractions of bacterial, protozoal, and dietary origin in omasal digesta were estimated with the AA profile approach proposed by Shabi et al. (2000) using the Standard Linear program in Premium Solver for Excel 2000 as described by Reynal et al. (2003). The estimated proportions, together with digesta flow measurements, were used to compute flows of NAN fractions from FAB, PAB, protozoa, and dietary origin at the omasal canal. Despite being statistically significant, differences in the concentrations of 11 AA between FAB and PAB were numerically small. Consequently, most of the solutions from linear programming optimized the objective function (minimum differences between actual and predicted AA flows using a nonnegative constraint) when all bacterial AA originated from either FAB or PAB. Therefore, the average AA composition of FAB and PAB for each cow in each period was used in the model to estimate the proportions of N from total bacterial, protozoal, and dietary origin in omasal digesta.

Statistical Analyses

Data used to assess treatment effects were analyzed using Proc Mixed in SAS (SAS Institute, 1999-2000). The following model was fit for all variables to assess treatment effects:

$$Y_{ijkl} = \mu + S_i + P_j + Vk_{(i)} + T_l + ST_{il} + E_{ijkl},$$

where Y_{ijkl} = dependent variable, μ = overall mean, S_i = effect of square i, P_j = effect of period j, $Vk_{(i)}$ = effect of cow k (within square i), T_l = effect of treatment l, ST_{il} = interaction between square i and treatment l, and E_{ijkl} = residual error. All terms were considered fixed, except for $Vk_{(i)}$ and E_{ijkl}, which were considered random. The interaction term ST_{il} was removed from the model when $P > 0.25$. Differences between least square means were reported only if the F-test for treatment was significant at $\alpha = 0.05$. Because dietary RDP concentrations among treatments were not equally spaced, linear, quadratic, and cubic effects of RDP were tested by partitioning the degrees of freedom for diet into single degree of freedom variables corresponding to linear, quadratic, and cubic effects. Data used to assess the effects of microbial marker on microbial and nonmicrobial NAN

flows, as well as data used to determine differences in AA composition among bacterial isolates were analyzed using the Proc Mixed procedure of SAS (SAS Institute, 1999-2000) with the following split-plot model:

$$Y_{ijklm} = \mu + S_i + P_j + Vk_{(i)} + T_l + ST_{il} + E1_{ijkl} + M_m + VM_{km} + SM_{im} + PM_{jm} + TM_{lm} + E2_{ijklm},$$

where Y_{ijklm} = dependent variable, μ = overall mean, S_i = effect of square i, P_j = effect of period j, $Vk_{(i)}$ = effect of cow k (within square i), T_l = effect of treatment l, M_m = effect of microbial marker or isolate m, $E1_{ijkl}$ and $E2_{ijklm}$ = whole-plot and subplot errors, VM_{km} = cow by microbial marker/isolate interaction, SM_{im} = square by microbial marker/isolate interaction, PM_{jm} = period by microbial marker/isolate interaction, and TM_{lm} = treatment by microbial marker/isolate interaction. All terms were considered fixed, except for $Vk_{(i)}$, $E1_{ijkl}$ and $E2_{ijklm}$, which were considered random. The interaction term ST_{il} was removed from the model when $P > 0.25$. Differences between least square means were reported only if the *F*-test for microbial marker was significant at $\alpha = 0.05$. For all variables measured except Ile the interaction term TM_{lm} was not significant at $\alpha = 0.05$. Therefore, least square means of the effects of microbial marker on microbial and nonmicrobial NAN flows and least square means of the AA profiles of each microbial fraction were reported across diets.

RESULTS AND DISCUSSION

Analysis of Purines and PD

Mean (±SD) recoveries of A and G added as yeast RNA to samples of FP, SP+LP, FAB, PAB, and protozoa were, respectively, 103.4% (±5.5) and 100.6% (±5.6); 98.6% (±7.2) and 97.7% (±6.6); 101.6% (±5.8) and 102.2% (±7.1); 97.2% (±4.2) and 97.6% (±3.6); and 99.0% (±4.1) and 98.7% (±3.2). Mean coefficients of variation between replicates were 3.3, 3.5, 3.4, 3.1, and 3.6% for FP, SP+LP, FAB, PAB, and protozoa, respectively.

If dietary purines are completely degraded in the rumen, purines of microbial origin flowing out of the rumen that are associated with fluid and particulate phases of digesta should have G:A ratios similar to those found in FAB and PAB isolates. Averaged across diets, G:A ratios in SP+LP and PAB were, respectively, 1.68 and 1.67, suggesting that purines associated with the particulate phase of digesta were of microbial origin only. However, G:A ratio of the FP averaged 5.70 and was 4.6 times greater than that in FAB, which had a mean G:A ratio of 1.25. A possible explanation for this discrepancy was the elution of other compounds that absorbed at 260 nm at the same retention time as G. To verify the purity of the A and G peaks, HPLC eluants from these peaks were collected from 4 samples each of FP, SP+LP, FAB, PAB, and protozoa and from A and G standards, and the UV spectra were compared (Figure 1). Spectra for A from sample hydrolysates closely paralleled those from the A standard. However, spectra for G from FP, FAB, and protozoa did not closely follow the pattern of G standards, suggesting coelution of other compounds with G. To resolve G from the unknown compound(s), the 30-min linear gradient from 0 to 100% solvent B (Makkar and Becker, 1999) was replaced by a 30-min linear gradient from 0 to 10% solvent B followed by a 30-min linear gradient from 10 to 100% solvent B. A second peak eluted approximately 20 s before G in samples of digesta and microbial isolates. The unknown compound was identified as hypoxanthine (**HX**), a metabolite of adenine, by comparing the retention time and the spectra of its peak to an HX standard (Figure 2). Extinction coefficients of G and HX were measured in 0.1 m*M* solutions of these compounds in a 50:50 mixture of HPLC buffers A and B (buffer proportions at time of coelution of G and HX) at room temperature using a quartz cuvette of 1-cm path-length at 254 nm in a spectrophotometer. Extinction coefficients for G and HX were 8231 and 8112 $M^{-1}cm^{-1}$, respectively. Based on the similarity of their extinction coefficients, the sum of G and HX concentrations were calculated from the area under the peak corresponding to their coelution at 16.1 min using the HPLC method of Makkar and Becker (1999).

Xanthine (**X**), a metabolite of both A and G, was also present in all samples. Xanthine and A eluted at 17.2 and 20.5 min and the assessment of their peak purity by UV spectra revealed no interfering compounds. When averaged across diets, X concentrations in microbial isolates (FAB, PAB, and protozoa), FP, and SP+LP accounted for 32, 37, and 28% of TP concentrations (Table 2). Concentrations of A and X (μmol/g of DM) were negatively correlated in FAB [X = 87 (±10; $P < 0.01$) – 1.46 (±0.29; $P < 0.01$) A; R = 0.55; $P < 0.01$], PAB [X = 70 (±5; $P < 0.01$) – 1.12 (±0.17; $P < 0.01$) A; R = 0.66; $P < 0.01$), and protozoa [X = 18 (±4; $P < 0.01$) – 1.14 (±0.50; $P = 0.03$) A; R = 0.42; $P = 0.03$]. This suggests that A in microbial isolates had been partially degraded to HX and subsequently to X during isolation. Omasal digesta samples were held on ice for approximately 4 h before isolation of bacteria and protozoa by differential centrifugation. During this time, bacterial cell lysis and enzymatic degradation of bacterial nucleic acids may have occurred. On the other hand, X concentrations in FP and SP+LP were not significantly correlated with A concentrations. Because omasal samples used for estimating flows of digesta phases were immediately frozen after collection, purine metabolites in these digesta

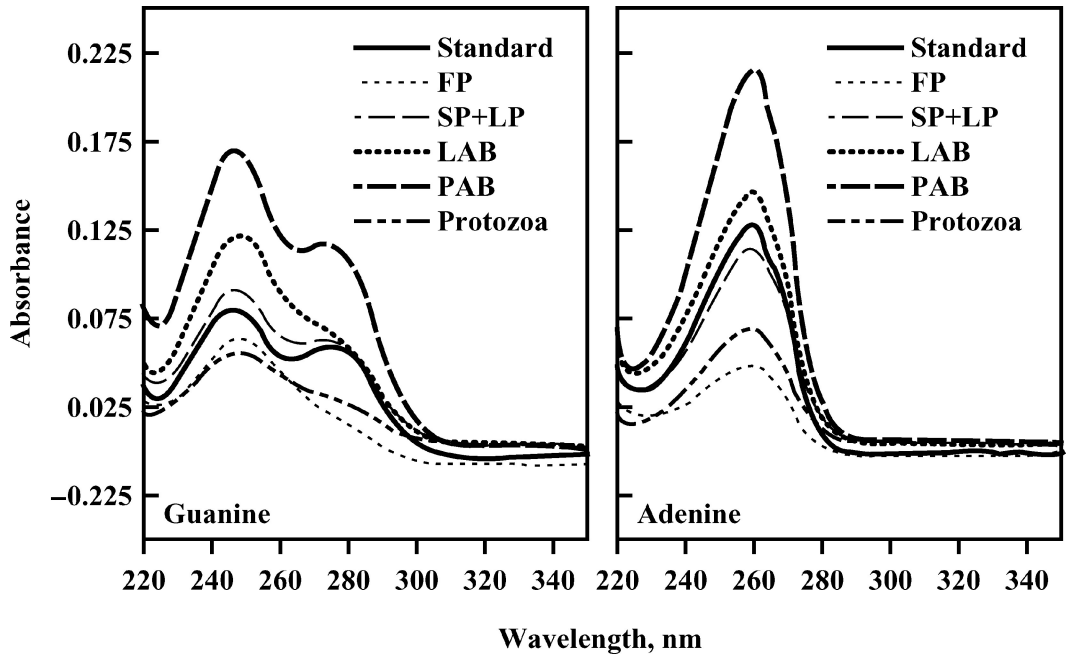

Figure 1. Ultraviolet spectra of HPLC eluants corresponding to adenine and guanine peaks in standard solutions, omasal fluid (FP), and particle (SP+LP) digesta phases, particle- (PAB) and fluid-associated bacteria (FAB), and protozoal isolates.

samples may have partly originated from incomplete degradation of dietary purines in the rumen. Vicente et al. (2004) reported that the proportion of duodenal purines of nonmicrobial origin, estimated from ^{15}N enrichment of purine-N in heifers supplemented with different protein sources, averaged 34% across diets. However, the origin of purines and their metabolites was not determined in the present study. Therefore, the purine metabolites X and HX present in both digesta samples and microbial isolates were assumed to be of microbial origin only and the sum of the purines and their metabolites was used as the microbial marker defined as TP.

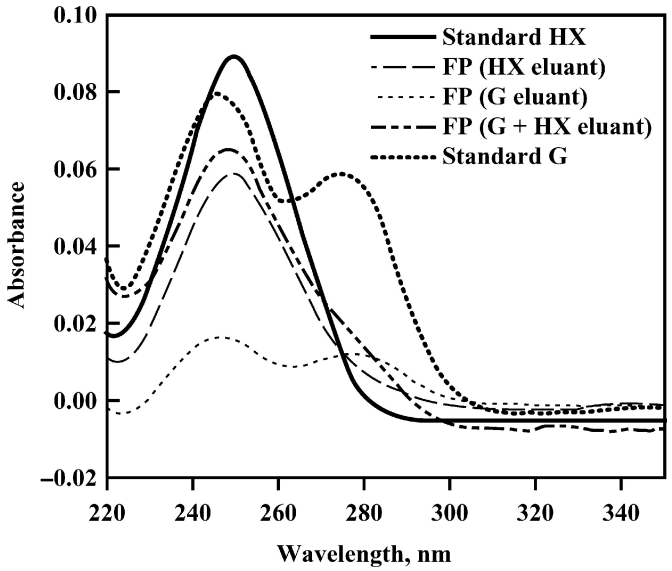

Figure 2. Ultraviolet spectra of HPLC eluants corresponding to guanine (G) and hypoxanthine (HX) peaks in standard solutions, omasal fluid phase (FP) using a modified HPLC method, and of G plus HX co-eluting after HPLC separation using the Makkar and Becker (1999) method.

Allantoin determination in urine samples was based on the Rimini-Shryver reaction (Vogels and van der Grift, 1970). Although used extensively for allantoin analysis, this reaction is not specific for allantoin and other compounds such as glyoxylic acid may interfere with allantoin determination (Chen et al., 1996). However, allantoin concentrations in sheep urine measured using Rimini-Shryver's colorimetry were highly correlated to those measured by spectrophotometric detection after HPLC separation. Resines et al. (1993) reported a correlation of 96% whereas Lindberg and Jansson (1989) reported a correlation of 99% with the intercept and slope of the regression of allantoin concentration by colorimetry on that by HPLC analysis of –0.09 m*M* and 1.03, respectively, over a range of 0.4 to 14 m*M* allantoin. Using the colorimetric method, recovery of allantoin added to sheep urine averaged 102% and coefficients of variation ranged from 1.7 to 5.6% (Lindberg and Jansson, 1989). Although these comparisons were made using sheep urine, it can be

Table 2. Effect of dietary RDP on NAN, TP concentration, NAN:TP ratio, and ^{15}N APE in omasal bacteria, protozoa, and digesta samples.

	Diet[2]					$P > F$[3]		
Item[1]	A 13.2	B 12.3	C 11.7	D 10.6	SE	RDP	Linear	Quadratic
NAN, % of OM								
FAB	9.74[a]	9.92[a]	9.29[ab]	8.87[b]	0.09	0.05	0.02	0.34
PAB	9.40[ab]	9.60[a]	9.25[bc]	9.08[c]	0.09	<0.01	<0.01	0.01
Protozoa	2.97	3.07	3.12	3.22	0.23	0.86	0.40	0.97
NAN, % of DM								
FAB	7.75[a]	7.78[a]	7.30[ab]	6.98[b]	0.19	<0.01	<0.01	0.45
PAB	7.99[ab]	8.15[a]	7.87[bc]	7.72[c]	0.09	<0.01	<0.01	0.04
Protozoa	2.86	2.95	3.01	3.07	0.22	0.89	0.45	0.88
FP	4.46[a]	4.32[ab]	4.16[b]	3.91[c]	0.17	<0.01	<0.01	0.41
SP+LP	4.40[b]	4.64[a]	4.51[ab]	4.60[a]	0.08	0.05	0.16	0.27
Purines and purine metabolites								
TP, μmol/g of DM								
FAB	124[a]	125[a]	114[b]	108[c]	3	<0.01	<0.01	0.34
PAB	116[b]	125[a]	111[bc]	105[c]	3	<0.01	<0.01	0.03
Protozoa	29.5	28.2	24.6	32.1	2.6	0.18	0.47	0.07
FP	51.4	51.5	51.3	46.6	1.9	0.07	0.03	0.17
SP+LP	33.6[a]	34.1[a]	31.0[b]	30.1[b]	1.3	<0.01	<0.01	0.73
Adenine, μmol/g of DM								
FAB	35.4	35.0	33.7	31.3	1.6	0.16	0.05	0.63
PAB	30.2	31.4	29.3	27.1	2.0	0.45	0.18	0.52
Protozoa	8.06	7.48	7.89	8.19	0.56	0.79	0.72	0.46
FP	5.53[ab]	5.96[a]	4.61[b]	4.87[b]	0.4	0.04	0.09	0.99
SP+LP	8.84[ab]	9.09[a]	8.15[b]	8.01[b]	0.43	0.04	0.03	0.74
Guanine + hypoxanthine, μmol/g of DM								
FAB	45.4[ab]	48.1[a]	44.1[ab]	41.5[b]	1.6	0.02	0.03	0.21
PAB	48.7	51.5	47.3	44.5	2.5	0.17	0.12	0.42
Protozoa	11.5	11.0	10.4	12.4	0.8	0.25	0.37	0.10
FP	25.3	25.8	27.3	25.6	1.2	0.67	0.86	0.44
SP+LP	14.8	15.2	14.4	13.9	0.7	0.29	0.17	0.55
Xanthine, μmol/g of DM								
FAB	42.0	41.2	36.5	33.4	3.0	0.15	0.24	0.89
PAB	36.7	42.4	34.6	33.8	3.2	0.23	0.40	0.40
Protozoa	9.47	9.59	6.41	11.7	1.6	0.12	0.41	0.09
FP	20.6[a]	19.8[a]	19.4[a]	16.7[b]	1.2	0.03	<0.01	0.40
SP+LP	9.93[a]	9.85[a]	8.36[b]	8.22[b]	0.51	<0.01	<0.01	0.84
NAN:TP ratios (g/mmol)								
FAB	0.63	0.63	0.64	0.66	0.02	0.75	0.67	0.74
PAB	0.70[b]	0.66[c]	0.72[ab]	0.74[a]	0.02	<0.01	<0.01	0.05
Protozoa	1.02	1.04	1.08	1.02	0.04	0.40	0.78	0.16
^{15}N APE								
FAB	0.037[b]	0.037[b]	0.040[b]	0.051[a]	0.003	<0.01	<0.01	<0.01
PAB	0.032[c]	0.032[c]	0.036[b]	0.045[a]	0.002	<0.01	<0.01	<0.01
Protozoa	0.031[b]	0.029[b]	0.034[b]	0.039[a]	0.002	<0.01	<0.01	0.03
FP	0.032[c]	0.032[c]	0.036[b]	0.045[a]	0.002	<0.01	<0.01	<0.01
SP+LP	0.018[b]	0.017[b]	0.018[b]	0.022[a]	0.001	<0.01	<0.01	<0.01

[a,b,c]Least squares means within the same row without a common superscript differ ($P < 0.05$).

[1]APE = Atom-percent enrichment, FAB = fluid-associated bacteria, FP = omasal fluid phase, PAB = particle-associated bacteria, SP+LP = omasal small plus large particle phases, TP = total purines.

[2]Diets and their corresponding RDP concentrations (in parentheses, as % of DM) measured in vivo.

[3]Probability of a significant effect of RDP or of a linear or quadratic effect of RDP concentration in the diet.

speculated that colorimetric detection of allantoin in cattle urine would yield similarly reliable results.

Chemical Composition of Digesta Samples and Microbial Isolates

Decreasing dietary RDP from 13.2 to 10.6% of DM resulted in significant linear decreases in the concentration of TP (A + G + X + HX) in FAB and digesta samples, of A in FAB and SP+LP, of G plus HX in FAB, and of X in FP and SP+LP (Table 2). Dietary RDP had linear and quadratic effects on TP concentration in PAB, with quadratic maxima corresponding to 12.5% RDP. Although NAN concentrations in both DM and OM of bacterial isolates decreased linearly with decreasing RDP, NAN:TP ratios in PAB increased linearly from 0.70 to 0.74. However, NAN:TP ratios in FAB and protozoa were not affected by diet and averaged 0.62 and

1.04, respectively. The NAN:TP ratios of FAB and PAB were lower than reported averages of, respectively, 1.20 and 1.65 (Carro and Miller, 2002), and 1.04 and 1.45 (Rodríguez et al., 2000), similar to those of 0.58 and 0.82 (Martin-Orué et al., 1998), and greater than averages of 0.50 and 0.59 (Pérez et al., 1997a) and 0.49 and 0.61 (Vicente et al., 2004), obtained when TP were determined using the HPLC method of Balcells et al. (1992). When expressed as a percentage of OM, concentrations of NAN in bacterial isolates ranged from 8.87 to 9.92% for FAB and from 9.08 to 9.60% for PAB and were within published ranges (Cecava et al., 1990; Pérez et al., 1997a; Rodríguez et al., 2000). However, protozoal NAN ranged from 2.97 to 3.22% of OM, which was substantially lower than reported concentrations of 8.0 to 8.6% (Volden et al., 1999b), 7.4 to 10.4% (Ahvenjärvi et al., 2002), 6.18 to 6.42% (Firkins et al., 1987a), and 4.2 to 5.2% (Martin et al., 1994). Purine content, NAN content, and N:purine ratio in protozoal isolates averaged 19.3 μmoles of purines/g of DM, 3.1% NAN (OM basis), and 1.45 g/mmol, which were, respectively, 27, 37 and 126% of the values reported by Volden et al. (1999b) using an HPLC method for purine analysis. This suggested that protozoal samples might have been contaminated with feed particles, resulting in proportionally higher dilution of purines than N in protozoal pellets. Because N from feeds would not be enriched with ^{15}N, contamination with feed N would have depressed the ^{15}N APE in protozoal isolates. However, enrichment of protozoa was, as expected, only slightly lower than that of FAB and PAB (Table 2). Therefore, samples were probably contaminated with non-nitrogenous compounds (e.g., sucrose added during isolation) without affecting N:purine ratios or ^{15}N APE.

Dietary RDP had linear and quadratic effects on ^{15}N APE of all microbial and digesta fractions, with the highest enrichment corresponding to the lowest RDP concentration and, therefore, the lowest dilution of infused $^{15}NH_3$ with dietary NH_3. The ^{15}N APE of the FAB isolates were, on average, 14% higher than those of PAB. Higher enrichment of FAB has been widely reported from in vivo, in vitro, and in situ studies (Martin et al., 1994; Pérez et al., 1996b; Ahvenjärvi et al., 2002) and may be attributed to greater use of NH_3 by FAB than by PAB (Pérez et al., 1996b; Carro and Miller, 1999). Protozoa become indirectly enriched with ^{15}N via bacterial predation and their ^{15}N APE will be lower than bacteria as a result of engulfment of unenriched dietary protein. In the present study, protozoal:bacterial enrichment ratios averaged 0.86. This ratio was higher than reported ratios of 0.75 (Cecava et al., 1991), 0.69 (Firkins et al., 1987a), 0.63 (Hristov and Broderick, 1996), and 0.40 (Ahvenjärvi et al., 2002). However, $^{15}NH_3$ was infused for a total of 158 h in our experiment, which may have allowed time for greater relative enrichment of protozoa.

Although dietary RDP had significant effects on the concentrations of 2 individual AA in FAB, 3 in PAB, and 2 in protozoa (Tables 3, 4, and 5), relative differences among treatments were numerically small. For FAB isolates, the proportions of Asp and Ser decreased linearly from 12.2 to 12.0% and from 4.49 to 4.38% of total AA ($P < 0.05$) for diets A to D, respectively (Table 3). For PAB isolates, decreasing RDP from diets A to D had a quadratic effect on the proportion of Ala (with maxima at 12.3% RDP; $P = 0.02$) and a negative linear effect on the proportion of Trp (from 1.47 to 1.40% of total AA; $P = 0.01$) (Table 4). Proportions of Ser in protozoal protein decreased linearly from 3.83 to 3.59% of total AA for diets A to D, respectively (Table 5). These results are in agreement with Korhonen et al. (2002), Martin et al. (1996), Volden and Harstad (1998), and Volden et al. (1999a), who reported significant but numerically small dietary effects on the concentrations of some AA in microbial isolates.

Because interactions between microbial isolates and dietary treatments were only significant for Ile ($P = 0.03$), the AA profiles of FAB, PAB, and protozoa were compared across diets (Table 6). Concentrations of 6 essential (Arg, Leu, Lys, Met, Phe, and Trp) and 6 nonessential AA (Ala, Cys, Glu, Gly, Pro, and Tyr) differed significantly between FAB and PAB. Most notably, concentrations of Met and Trp were 24 and 11% higher, respectively, and concentration of Lys was 6% lower in FAB compared with PAB ($P < 0.05$). Higher concentrations of Ala, Gly, and Tyr and lower concentrations of Arg, Leu, and Phe in FAB compared with PAB were also reported previously (Martin et al., 1996; Volden and Harstad, 1998; Volden et al., 1999a,b; Rodríguez et al., 2000; Korhonen et al., 2002). Concentrations of 15 AA in FAB and 17 AA in PAB were significantly different than in protozoa. It is worth noting that Lys concentration in protozoa was 22% higher than PAB and 29% higher than FAB, Met in protozoa was 137% higher than PAB and 92% higher than FAB, whereas Trp in protozoa was 51% of that in FAB and 57% of that in PAB ($P < 0.05$). Higher concentrations of Lys in protozoa were reported by Martin et al. (1996) and Volden et al. (1999) whereas higher concentrations of both Lys and Met were also reported by Korhonen et al. (2002). Therefore, these results indicated that accurate estimates of microbial AA flow from the rumen require measurement of the AA profiles of both bacteria and protozoa and their relative contribution to total AA flow.

Effect of Dietary RDP on Microbial NAN Flows

Total microbial NAN flows estimated using TP, ^{15}N, AA profiles, and PD as the microbial markers decreased

Table 3. Effect of dietary RDP on AA composition of particle-associate bacteria.

Amino acid	Diet[1] A (13.2)	B (12.3)	C (11.7)	D (10.6)	SE	$P > F^2$ RDP	Linear	Quadratic
	(%)							
Essential								
Arg	4.18	4.23	4.19	4.23	0.05	0.73	0.65	0.89
His	1.60	1.61	1.59	1.59	0.02	0.78	0.66	0.72
Ile	5.98	5.98	5.89	5.98	0.08	0.83	0.88	0.58
Leu	8.03	8.02	8.05	8.10	0.05	0.58	0.25	0.65
Lys	6.10	6.11	5.93	5.89	0.09	0.18	0.07	0.90
Met	3.00	2.94	2.79	3.17	0.23	0.69	0.72	0.33
Phe	4.39	4.34	4.38	4.37	0.07	0.96	0.99	0.78
Thr	5.50	5.51	5.51	5.44	0.03	0.29	0.18	0.19
Trp	1.63	1.62	1.61	1.62	0.03	0.97	0.79	0.87
Val	5.21	5.30	5.23	5.27	0.04	0.44	0.43	0.67
Nonessential								
Ala	7.28	7.36	7.30	7.28	0.05	0.52	0.88	0.53
Asp	12.2[a]	12.2[a]	12.2[a]	12.0[b]	0.1	<0.01	<0.01	0.31
Cys	2.85	2.79	3.06	2.92	0.09	0.10	0.21	0.50
Glu	13.9	13.9	14.1	14.0	0.1	0.35	0.32	0.88
Gly	3.36	3.42	3.39	3.39	0.03	0.61	0.62	0.52
Pro	3.38	3.32	3.44	3.35	0.06	0.49	0.96	0.78
Ser	4.49	4.43	4.45	4.38	0.03	0.07	0.04	0.96
Tyr	4.96	5.00	4.94	5.03	0.05	0.55	0.37	0.56

[a,b]Least squares means within the same row without a common superscript differ ($P < 0.05$).

[1]Diets and their corresponding RDP concentrations (in parentheses, as % of DM) measured in vivo.

[2]Probability of a significant effect of RDP or of a linear or quadratic effect of RDP level in the diet.

Table 4. Effect of dietary RDP on AA composition of particle-associated bacteria.

Amino acid	Diet[1] A (13.2)	B (12.3)	C (11.7)	D (10.6)	SE	$P > F^2$ RDP	Linear	Quadratic
	(%)							
Essential								
Arg	4.39	4.40	4.39	4.35	0.07	0.89	0.52	0.71
His	1.62	1.61	1.60	1.59	0.02	0.79	0.30	0.91
Ile	5.85	5.83	5.88	5.85	0.05	0.90	0.88	0.95
Leu	8.26	8.22	8.26	8.21	0.04	0.64	0.55	0.75
Lys	6.36	6.42	6.35	6.45	0.06	0.57	0.53	0.64
Met	2.41	2.48	2.52	2.46	0.07	0.12	0.31	0.81
Phe	4.49	4.45	4.48	4.51	0.04	0.50	0.40	0.27
Thr	5.46	5.54	5.45	5.47	0.03	0.13	0.77	0.68
Trp	1.47[a]	1.48[a]	1.47[a]	1.40[b]	0.02	0.02	0.01	0.07
Val	5.21	5.32	5.24	5.17	0.05	0.21	0.37	0.11
Nonessential								
Ala	6.73[bc]	6.93[a]	6.76[b]	6.66[c]	0.08	<0.01	0.16	0.02
Asp	11.9	11.9	11.9	11.9	0.1	0.88	0.65	0.54
Cys	3.44	3.35	3.50	3.58	0.09	0.14	0.07	0.31
Glu	14.2	14.1	14.1	14.2	0.1	0.42	0.52	0.20
Gly	5.23	5.35	5.27	5.23	0.04	0.11	0.64	0.06
Pro	3.65	3.62	3.57	3.65	0.06	0.81	0.95	0.49
Ser	4.47	4.39	4.39	4.38	0.04	0.30	0.17	0.56
Tyr	5.84	5.79	5.88	5.90	0.04	0.11	0.06	0.38

[a,b,c]Least squares means within the same row without a common superscript differ ($P < 0.05$).

[1]Diets and their corresponding RDP concentrations (in parentheses, as % of DM) measured in vivo.

[2]Probability of a significant effect of RDP or of a linear or quadratic effect of RDP level in the diet.

Table 5. Effect of dietary RDP on AA composition of protozoa.

Amino acid	Diet[1] A (13.2)	B (12.3)	C (11.7)	D (10.6)	SE	$P > F^2$ RDP	Linear	Quadratic
	(%)							
Essential								
Arg	4.06	4.09	4.14	4.13	0.05	0.38	0.12	0.42
His	1.44	1.46	1.47	1.46	0.02	0.68	0.37	0.39
Ile	6.06	6.02	5.92	5.98	0.07	0.49	0.28	0.36
Leu	7.80	7.85	7.84	7.84	0.09	0.95	0.73	0.63
Lys	7.96	7.88	7.60	7.58	0.27	0.43	0.11	0.77
Met	5.83	5.84	5.84	5.52	0.32	0.78	0.45	0.59
Phe	4.38	4.55	4.45	4.51	0.07	0.44	0.37	0.50
Thr	4.86	5.15	5.07	5.16	0.09	0.13	0.06	0.28
Trp	0.82	0.82	0.80	0.83	0.02	0.69	0.84	0.51
Val	4.52	4.49	4.51	4.49	0.10	0.99	0.81	0.93
Nonessential								
Ala	4.91	4.77	4.84	4.88	0.19	0.84	0.99	0.44
Asp	12.2	12.4	12.3	12.7	0.24	0.29	0.10	0.52
Cys	4.82	4.53	4.84	4.38	0.22	0.42	0.27	0.71
Glu	14.4	14.4	14.5	14.7	0.1	0.25	0.07	0.85
Gly	4.21	4.22	4.24	4.27	0.08	0.86	0.41	0.90
Pro	3.29	3.29	3.35	3.41	0.11	0.43	0.13	0.74
Ser	3.83	3.63	3.71	3.59	0.10	0.17	0.04	0.55
Tyr	4.63	4.56	4.59	4.57	0.02	0.15	0.11	0.30

[1]Diets and their corresponding RDP concentrations (in parentheses, as % of DM) measured in vivo.
[2]Probability of a significant effect of RDP or of a linear or quadratic effect of RDP level in the diet.

linearly with decreasing dietary RDP by 11, 18, 21, and 23%, respectively ($P < 0.05$; Table 7). As availability of N in the rumen decreased, efficiency of microbial protein synthesis decreased linearly from 32.3 to 28.0 (estimated using ^{15}N) and from 25.5 to 19.8 (estimated using AA profiles) g of NAN per kg of OM truly digested in the rumen, suggesting that the limiting factor for microbial growth was not digestible OM but rather availability of RDP (Reynal and Broderick, 2005). However, it was not possible to determine which RDP source or sources were limiting microbial growth. Decreasing dietary RDP from 13.2 to 10.6% of DM resulted in linear decreases in ruminal ammonia-N (from 12.3 to 5.7 mg/dL; $P < 0.01$) and free AA (from 4.9 to 3.4 m*M*; $P < 0.01$). Ruminal ammonia concentrations above the microbial requirement of 5 mg/dL determined in vitro (Satter and Slyter, 1974; Russell and Strobel, 1987) might be needed for maximal microbial yield in vivo (Hume et al., 1970; Allen and Miller, 1976; Kang-Meznarich and Broderick, 1980; Balcells et al., 1993) and for maximal degradation rates of feeds incubated in situ (Erdman et al., 1986; Odle and Schaefer, 1987). On the other hand, growth rates and yields of ruminal microorganisms have been shown to be stimulated by AA and peptides in vivo (Chikunya et al., 1996) and in vitro (Argyle and Baldwin, 1989; Carro and Miller, 1999), and by degraded true protein in vitro (Hristov and Broderick, 1994). Although ruminal peptide concentrations were not measured in our study, it may be speculated that peptide availability increased with increasing dietary protein degradability. Further discussion on microbial NAN yield responses to dietary RDP is presented in the companion paper (Reynal and Broderick, 2005).

Table 6. Amino acid profiles of microbial isolates.[1]

Amino acid	FAB	PAB	Protozoa	SEM
	AA, % of total AA			
Essential				
Arg	4.21[b]	4.38[a]	4.11[b]	0.04
His	1.60[a]	1.60[a]	1.46[b]	0.01
Ile	5.93[b]	5.85[b]	6.00[a]	0.04
Leu	8.05[b]	8.24[a]	7.83[b]	0.03
Lys	6.02[c]	6.38[b]	7.76[a]	0.10
Met	2.99[b]	2.42[c]	5.75[a]	0.13
Phe	4.37[b]	4.48[a]	4.47[a]	0.04
Thr	5.48[a]	5.48[a]	5.06[b]	0.03
Trp	1.61[a]	1.45[b]	0.82[c]	0.01
Val	5.25[a]	5.24[a]	4.50[b]	0.05
Nonessential				
Ala	7.31[a]	6.78[b]	4.85[c]	0.10
Asp	12.1[b]	11.9[b]	12.4[a]	0.1
Cys	2.91[c]	3.47[b]	4.64[a]	0.08
Glu	14.0[c]	14.2[b]	14.5[a]	0.05
Gly	5.38[a]	5.28[b]	4.23[c]	0.04
Pro	3.38[b]	3.62[a]	3.34[b]	0.04
Ser	4.44[a]	4.40[a]	3.69[b]	0.04
Tyr	4.97[a]	4.85[b]	4.59[c]	0.02

[a,b,c]Within a row, means without a common superscript differ ($P < 0.05$).
[1]FAB = Fluid-associated bacteria, PAB = particle-associated bacteria.

Table 7. Microbial NAN flows at the omasal canal measured using TP, ^{15}N, AA profiles, and PD as microbial markers.

	Diet[1]					$P > F^2$		
Item[3]	A (13.2)	B (12.3)	C (11.7)	D (10.6)	SE	RDP	Linear	Quadratic
Omasal NAN flow, g/d	684^{a}	664^{a}	653^{ab}	615^{b}	26	0.03	<0.01	0.55
Using TP as marker								
Microbial NAN flows								
FAB, g/d	161	160	166	149	11	0.58	0.37	0.47
PAB, g/d	250	236	231	221	17	0.12	0.03	0.75
Total, g/d	415	397	398	369	23	0.12	0.02	0.90
% of total NAN flow	60.9	59.3	60.8	59.9	1.9	0.74	0.49	0.69
NANMN flow								
g/d	260	270	255	244	12	0.26	0.36	0.35
% of total NAN flow	39.1	40.7	39.2	40.1	1.8	0.74	0.49	0.69
Microbial efficiency, g of NAN/kg of OMTDR	28.4	27.1	26.4	26.8	1.0	0.50	0.17	0.27
Using ^{15}N as marker								
Microbial NAN flows								
FAB, g/d	199^{a}	189^{a}	191^{a}	167^{b}	10	0.05	0.04	0.45
PAB, g/d	271^{a}	249^{b}	233^{bc}	217^{c}	14	<0.01	<0.01	0.80
Total, g/d	470^{a}	438^{b}	425^{b}	384^{c}	18	<0.01	<0.01	0.71
Total, % of total NAN	69.6^{a}	65.8^{b}	65.1^{bc}	62.1^{c}	1.3	<0.01	<0.01	0.85
NANMN flow								
g/d	209	226	229	233	13	0.36	0.24	0.62
% of total NAN flow	30.4^{c}	34.2^{b}	34.9^{ab}	37.8^{a}	1.3	<0.01	<0.01	0.85
Microbial efficiency, g of NAN/kg of OMTDR	32.3^{a}	30.1^{b}	28.1^{c}	28.0^{c}	0.8	<0.01	<0.01	0.28
Using AA profiles as marker								
Microbial NAN flows								
FAB + PAB, g/d	390^{a}	393^{a}	339^{a}	241^{b}	34	<0.01	<0.01	0.07
Protozoa, g/d	3.7^{b}	14.3^{b}	8.4^{b}	70.7^{a}	13	<0.01	<0.01	0.03
Total, g/d	392^{ab}	407^{a}	348^{bc}	311^{c}	27	0.01	<0.01	0.34
Total, % of total NAN	55.5^{a}	56.3^{a}	50.7^{ab}	47.9^{b}	3.6	<0.01	<0.01	0.10
NANMN flow								
g/d	314	316	339	338	20	0.63	0.26	0.83
% of NAN	44.5^{bc}	43.7^{c}	49.3^{ab}	52.0^{a}	2.6	0.02	<0.01	0.79
Microbial efficiency, g of NAN/kg of OMTDR	25.5^{a}	24.4^{a}	21.0^{b}	19.8^{b}	1.2	<0.01	<0.01	0.80
Using PD as marker								
Total urine excretion, L/d	22.8	19.5	19.5	17.2	2.2	0.19	0.04	0.80
Allantoin excretion, mmol/d	460^{a}	343^{b}	358^{b}	317^{b}	29	<0.01	<0.01	0.10
Uric acid excretion, mmol/d	8.23	9.22	10.39	11.48	1.65	0.47	0.13	0.96
Total PD excretion, mmol/d	468	353	369	329	29	<0.01	<0.01	0.11
Microbial NAN flow,[4] g/d	436^{a}	299^{b}	319^{b}	271^{b}	35	<0.01	<0.01	0.11

[a,b,c]Least squares means within the same row without a common superscript differ ($P < 0.05$).

[1]Diets and their corresponding RDP concentrations (in parentheses, as % of DM) measured in vivo.

[2]Probability of a significant effect of RDP or of a linear or quadratic effect of RDP concentration in the diet.

[3]FAB = Fluid-associated bacteria, NANMN = nonammonia nonmicrobial N, OMTDR = OM truly digested in the rumen, PAB = particle-associated bacteria, PD = purine derivatives, TP = total purines.

[4]Estimated from urinary PD excretion of ruminally cannulated cows using equations from Vagnoni et al., 1997.

Comparison among Microbial Markers

When TP was used as the microbial marker, flows of NANMN expressed as both grams per day and percentage of total NAN flow were not affected by dietary RDP. This apparent lack of response of RUP flow to dietary changes in RUP and RDP disagreed with both literature reports and responses predicted by the NRC (2001) model. In the studies of Mansfield and Stern (1994) and Windschitl and Stern (1988), replacement of solvent soybean meal with lignosulfonate-treated soybean meal significantly increased NANMN flows from 351 to 371 g/d and from 130 to 207 g/d, respectively, when the SBM supplements supplied 40 to 52% of the CP in diets fed to dairy cows. Using the actual diet composition and DMI in this study, the NRC (2001) model predicted RUP flows should increase from 1426 to 2137 g/d, from diet A to diet D, respectively.

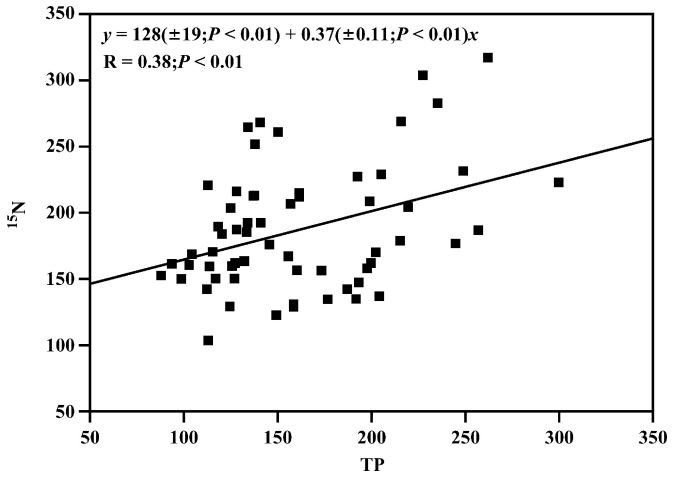

Figure 3. Correlation between fluid-associated bacteria—NAN flows at the omasal canal measured using ^{15}N and total purines (TP) as microbial markers.

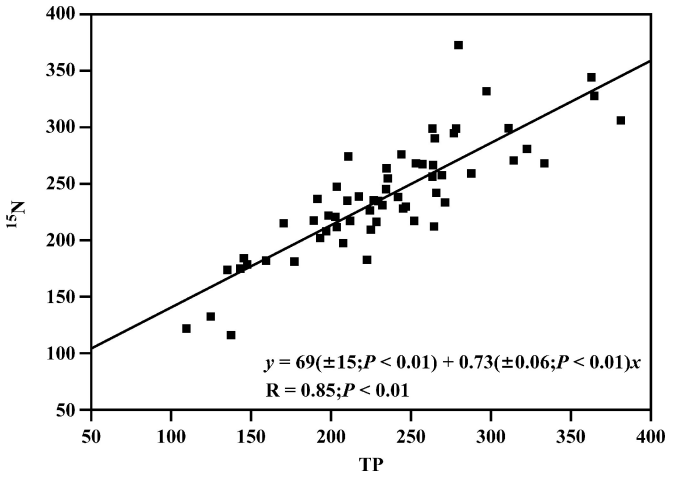

Figure 4. Correlation between particle-associated bacteria—NAN flows at the omasal canal measured using ^{15}N and total purines (TP) as microbial markers.

Flows of NANMN (expressed as % of total NAN flow) increased linearly from 30.4 to 37.8% when using ^{15}N and from 44.5 to 52.0% when using AA profiles as microbial markers (Table 7). Therefore, dietary NAN flow responses to changes in RUP estimated using ^{15}N and AA profiles as the microbial markers were in agreement with previously published studies (Windschitl and Stern, 1988; Mansfield and Stern, 1994) and NRC (2001) predictions.

The correlation between FAB-NAN flows estimated using TP and ^{15}N was poor (R = 0.38; $P < 0.01$; Figure 3), with flows estimated using TP being significantly lower than those estimated using ^{15}N (Table 8). However, flows of PAB-NAN estimated using TP were not significantly different from those estimated using ^{15}N (Table 8), with a correlation between markers of 0.85 ($P < 0.01$; Figure 4). If purines from dietary origin were not completely degraded in the rumen, they may have preferentially partitioned to the fluid fraction of digesta as was previously observed by Pérez et al. (1997b) and Vicente et al. (2004). Therefore, the lack of agreement between FAB flows based on TP and ^{15}N may have originated from the higher contribution of dietary purines to FP, compared with SP+LP. Flows of total microbial-NAN estimated using TP were not significantly different from those estimated using ^{15}N (Table 8) and the correlation between markers was intermediate between those for FAB and PAB (R = 0.69; $P < 0.01$; Figure 5). Similarly, Carro and Miller (2002) found a significant correlation between microbial flows estimated with TP (determined by HPLC) and ^{15}N (R = 0.88; $P < 0.01$) in semicontinuous fermenters. Moreover, the slope of the regression of TP on ^{15}N for microbial

Table 8. Comparison among microbial markers for quantifying microbial and nonmicrobial NAN flows at the omasal canal.

	Microbial marker[2]						
Item[1]	TP	^{15}N	AAp	PD	SEM	$P > F^3$	T × M[4] P-value
Microbial NAN flows, g/d							
FAB	160[b]	186[a]	—	—	5	<0.01	0.85
PAB	236	243	—	—	4	0.27	0.65
Total	398[a]	429[a]	360[b]	305[c]	13	<0.01	0.34
NANMN flows, g/d	255[b]	224[c]	326[a]	—	7	<0.01	0.75

[a,b,c]Least square means within the same row without a common superscript differ ($P < 0.05$).

[1]FAB = Fluid-associated bacteria, NANMN = nonammonia nonmicrobial N, PAB = particle-associated bacteria.

[2]AAp = AA Profiles, PD = purine derivatives, TP = total purines.

[3]Probability of a significant effect of microbial marker.

[4]Probability of a significant interaction between treatment and marker.

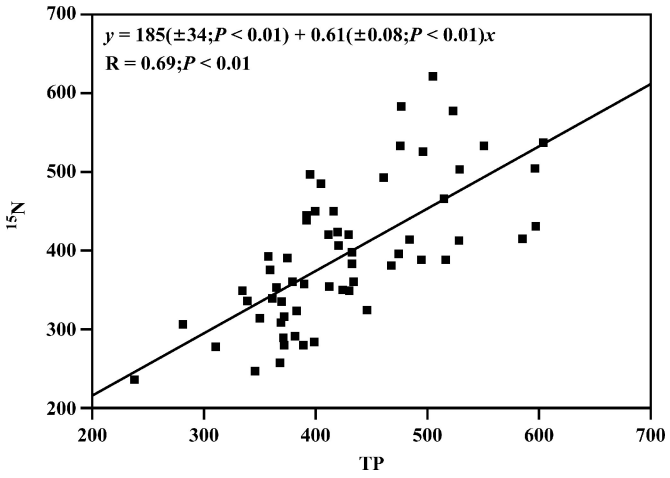

Figure 5. Correlation between total microbial NAN flows (fluid-associated bacteria—NAN plus particle-associated bacteria—NAN flows) at the omasal canal measured using ^{15}N and total purines (TP) as microbial markers.

NAN flow was 0.56, similar to the significant slope of 0.61 found in the present study (Figure 5). Therefore, as suggested by Carro and Miller (2002), significant intercepts and slopes different from 1 (Figures 3, 4, and 5) suggest that ^{15}N and TP responded differently to the dietary treatments (e.g., to varying extents of ruminal degradation of purines from different feedstuffs). On the other hand, FAB-NAN flows were significantly affected by treatments when ^{15}N but not TP was the microbial marker (Table 7), despite the lack of significant marker by treatment interactions (Table 8). Although PAB and total microbial NAN flow decreased linearly from diets A to D when estimated using TP as the microbial marker, the slopes of their respective regressions on dietary RDP (11.4 and 19.3 g per percentage unit RDP; $P < 0.01$) were substantially lower than those estimated using ^{15}N (20.9 and 32.6 g per percentage unit RDP; $P < 0.01$).

When Calsamiglia et al. (1996) regressed microbial NAN flows, estimated in continuous culture fermenters using TP (determined by the method of Zinn and Owens), on those estimated with ^{15}N, the regression coefficient and slope were 0.06 and 0.15, respectively, possibly due to the low precision of the TP method used. In the present study, differences between microbial flow estimates based on TP and ^{15}N may have been due, at least in part, to the higher variability of TP estimates: standard errors for FAB, PAB, and total microbial flows were, respectively, 11, 17, and 23 when using TP, and 10, 14, and 18 when using ^{15}N (Table 7). In the studies of Firkins et al. (1987a) and Cecava et al. (1991), treatment means for microbial N flows at the duodenum of steers were similar when estimated using either marker. However, no correlation between markers was reported. On the other hand, when individual purines were determined using an HPLC method, mean microbial N flows to the duodenum of sheep were lower than those estimated with ^{15}N (Pérez et al., 1996a). These inconsistencies between microbial N yields obtained using TP or ^{15}N could be due to: 1) lower precision of the Zinn and Owens (1986), (Calsamiglia et al., 1996), or HPLC methods of purine analysis compared with ^{15}N; 2) incomplete ruminal degradation of purines from different feedstuffs (Pérez et al., 1996b; Vicente et al., 2004); 3) unrepresentative isolation of the microbial preparations (Firkins et al., 1987a; Pérez et al., 1997a); or 4) disproportionate metabolism of nucleic acids released from microbial cells lysed intraruminally (Broderick and Merchen, 1992).

Purines reaching the duodenum of cattle are mainly of microbial origin and are extensively metabolized and excreted in the urine as allantoin and uric acid; thus, changes in microbial flow from the rumen should result in similar changes in the urinary excretion of PD (Chen et al., 1990; Balcells et al., 1991; Orellana Boero et al., 2001). Between 84% (Orellana Boero et al., 2001) and 86% (Vagnoni et al., 1997) of the exogenous purines infused postruminally in cows were recovered in the urine. Moreover, Martin-Orué et al. (2000) reported that urinary excretion of PD was highly correlated with duodenal flows of purines and closely reflected the relative differences among treatments in duodenal flows observed in heifers. Similarly, microbial N flows at the duodenum of cows fed at 100, 85, and 75% of ad libitum intake decreased linearly with decreasing feed allowance when estimated from either duodenal purine flow or from urinary PD excretion (González-Ronquillo et al., 2004). However, in the present study, relative microbial N responses to treatments were substantially different between direct and indirect approaches. Although microbial NAN flows estimated using TP decreased linearly ($P = 0.02$) from 415 to 369 g/d (slope = 19.3 g per percentage unit RDP; $P = 0.02$), those estimated from the urinary excretion of purine derivatives decreased linearly ($P < 0.01$) from 436 to 271 g/d (slope = 35.3 g per percentage unit RDP; $P < 0.01$; Table 7). Moreover, when averaged across diets, microbial N flows estimated from the flow of TP at the omasal canal were 31% higher than those estimated from urinary excretion of PD (401 vs. 305 g/d; $P < 0.01$; Table 8). Other authors have reported direct microbial flow estimations that were 48% higher (Martin-Orué et al., 2000) and 30% lower (González-Ronquillo et al., 2004) than indirect estimates based on PD excretion. The lack of agreement between direct and indirect estimates of microbial NAN flows averaged across treatments found in our study could be explained, at least in part, by

differences between the physiological conditions of the animals used to develop the equation of Vagnoni et al. (1997) (dry and late lactation cows) and those of the animals used in the present experiment (cows in early lactation). González-Ronquillo et al. (2003) reported that the proportion of duodenal purines recovered in the urine was significantly higher for cows in late lactation compared with early lactation (0.63 vs. 0.44, respectively). However, caution is needed when interpreting these results due to the small number of animals used (3 per lactation stage). Moreover, applying the equation of González-Ronquillo et al. (2003) for early-lactation cows in the present experiment yielded microbial NAN flows that were unrealistically high and ranged from 512 to 828 g/d. Averaged across diets, microbial NAN flows based on PD were 71% of those based on ^{15}N (305 vs. 429 g/d; $P < 0.01$; Table 8). However, the slopes from regressing dietary RDP on microbial NAN flows estimated using PD and ^{15}N were similar (35.3 and 32.6 g per percentage unit RDP; $P < 0.01$). This indicated that urinary excretion of PD estimated from spot urine samples were effective for detecting the relative differences among treatments in microbial NAN flow from the rumen.

The approach based on AA profiles yielded microbial NAN flows that were 90 and 84% of those estimated using, respectively, TP and ^{15}N, but 18% higher than PD-based estimates ($P < 0.01$; Table 8). Siddons et al. (1982) also observed that the proportion of microbial N in duodenal digesta of sheep was significantly lower when estimated using AA profiles compared with ^{15}N. When compared with TP (HPLC analysis), the use of AA profiles as microbial marker resulted in 22% higher microbial NAN flows at the omasal canal of lactating dairy cows (Reynal et al., 2003). The assumption that the AA profile of RUP is the same as that of dietary protein may not hold true. The AA profiles of feedstuffs incubated in situ were significantly altered by ruminal degradation (Erasmus et al., 1994; O'Mara et al., 1997). Therefore, differences between the AA profiles approach and other markers may have been partly due to the inaccuracy of this assumption. Although microbial flows averaged across diets differed significantly between ^{15}N and AA profiles, the estimated extent and direction of the changes across dietary treatments were in close agreement between these 2 methods (Table 7). The slopes from regressing dietary RDP on microbial NAN flows estimated using AA profiles and ^{15}N were, respectively, 33.7 and 32.6 g per percentage unit RDP ($P < 0.01$). Therefore, our results suggested that the use of AA profiles will yield accurate assessment of differences among treatments but may result in biased estimates of NAN flows of dietary and microbial origin.

Protozoal N may account for 35 to 66% of the microbial N within the rumen (Faichney et al., 1997). Despite the sequestration of protozoa in the rumen (Leng, 1982; Faichney, 1989), protozoal N may account for a significant proportion of the total N flowing to the intestines. Because TP:NAN ratios and ^{15}N APE in isolated protozoa were, on average, 65 and 86% of those in bacteria (Table 2), the use of bacteria alone as reference may have resulted in underestimation of microbial flow when using TP and ^{15}N as markers. The contribution of protozoal N to total N flow could be determined using an internal marker specific for protozoa. Although aminoethyl-phosphonic acid has been used as a protozoal marker, its presence has been detected in bacteria and feedstuffs (Ankrah et al., 1989; Horigane and Horiguchi, 1990). Because phosphatidyl choline was found in rumen protozoa but not in bacteria, and because dietary phosphatidyl choline was readily degraded in the rumen, it was suggested as a suitable protozoal marker (John and Ulyatt, 1984). Using this marker, John and Ulyatt (1984) estimated that protozoal N contributed up to 14% of total microbial N. Also using phosphatidyl choline, Robinson et al. (1996) estimated that protozoal N accounted for 13% of total duodenal N flow. Simultaneously solving equations for bacterial and protozoal contributions to duodenal NAN flow based on total purines and ^{15}N, Firkins et al. (1987a) estimated that protozoal N contributed 27% of the total duodenal N flow. In the present study, protozoal contributions to total NAN flows estimated using AA profiles were substantially lower than these reported values and were significantly higher for diet D (11.5%) compared with the other diets (mean 1.33% for diets A, B and C) (Table 7). A reliable marker specific for protozoa is needed to assess the accuracy of using AA profiles for estimating protozoal NAN flows from the rumen.

The lack of a standard method with which microbial flow estimates can be compared makes it difficult to assess the accuracy of the microbial markers tested. However, ^{15}N has several theoretical advantages over the other markers that may result in more precise and accurate estimates. Because ^{15}N from $^{15}NH_3$ should be evenly distributed throughout the microbial cell, loss of cytoplasmic contents during isolation would not affect microbial enrichments and flow estimations based on ^{15}N. On the contrary, microbial lysis during isolation could lower the purine:N ratios in microbial preparations, resulting in overestimation of microbial flows estimated using purines (Martin-Orué et al., 1998; Carro and Miller, 2002). Moreover, unless contamination occurs, protein leaving the rumen that is enriched in excess of natural abundance will be of microbial origin only, whereas some portion of the purines leaving the rumen can be of dietary origin (Pérez et al., 1996b;

Vicente et al., 2004). Furthermore, the excellent accuracy and precision of ^{15}N determination by isotope-ratio mass spectrometry (Broderick and Merchen, 1992) should result in greater precision of microbial flow estimates compared with the other markers. In the present study, use of ^{15}N yielded lower standard errors for all variables measured compared with the other markers. Therefore, based on theoretical and practical considerations, results based on ^{15}N were considered the most accurate in the present trial.

CONCLUSIONS

Dietary RDP concentration had significant effects on the AA profiles of FAB, PAB, and protozoa. Moreover, AA profiles and concentrations differed substantially between FAB and PAB and between both bacterial isolates and protozoa. Therefore, accurate estimates of microbial AA flow from the rumen require measurement of the AA profiles of both bacteria and protozoa and their relative contributions to total AA flow. The HPLC method used to assay total purines in digesta and microbial samples did not resolve G and HX and should be used with caution. Ruminal escape of dietary protein estimated using TP as microbial marker was in contradiction with published and predicted responses to changes in dietary RUP and RDP and, therefore, was considered biased. Estimates of microbial and dietary NAN flows at the omasal canal based on ^{15}N were in agreement with published and predicted values and were considered accurate. Although the use of AA profiles and PD may result in biased estimates of microbial NAN flow across diets, these markers accurately predicted the direction and extent of changes induced by dietary treatments. Based on our findings and on theoretical considerations, we recommend using $^{15}NH_3$ with continuous intraruminal infusion rather than using TP as the microbial marker. A reliable protozoal marker is needed to accurately estimate microbial NAN flows from the rumen.

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